Uncoupling oxidative phosphorylation with 2,4-dinitrophenol promotes development of the adhesion phenotype

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Objective: To determine the effect of uncoupling oxidative phosphorylation with 2,4-dinitrophenol (DNP) on adhesion phenotype development.
Design: Prospective experimental study.
Setting: Academic medical center.
Patient(s): Women undergoing laparotomy for pelvic pain from whom normal peritoneum and adhesions were excised to create primary cultures of normal peritoneal and adhesion fibroblasts.
Intervention(s): Treatment of normal peritoneal and adhesion fibroblasts isolated from the same patient(s) with or without 0.2 mM DNP for 24 hours.
Main Outcome Measure(s): Evaluation of adhesion phenotype markers type I collagen, vascular endothelial growth factor (VEGF), and hypoxia-inducible factor (HIF)-1α.
Result(s): In agreement with prior findings, adhesion fibroblasts exhibited significantly higher basal levels of type I collagen, VEGF, and HIF-1α compared with normal peritoneal fibroblasts. Treatment of normal peritoneal fibroblasts with DNP resulted in significant increases in type I collagen (10.2 ± 1.4 vs. 18.4 ± 1.9 fg/μg RNA) and VEGF (8.2 ± 1.1 vs. 13.7 ± 0.4 fg/μg RNA) over baseline. HIF-1α levels did not increase when normal peritoneal fibroblasts were treated with DNP.
Conclusion(s): The adhesion phenotype, which is normally expressed in response to hypoxia, is reproduced in a normoxic environment by uncoupling oxidative phosphorylation with DNP, as evidenced by an increase in type I collagen and VEGF. Acquisition of the adhesion phenotype was via a mechanism distinct from up-regulation of HIF-1α. These observations are consistent with the hypothesis that the adhesion phenotype represents a state of intracellular metabolic depletion. (Fertil Steril® 2012;97:729–33. ©2012 by American Society for Reproductive Medicine.)
Key Words: Postoperative adhesions, 2,4-dinitrophenol (DNP), oxidative phosphorylation, collagen, vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)

Injury to the peritoneum during surgery frequently results in the development of postoperative adhesions. Postoperative adhesions, which are nonanatomic fibrous connections between tissue surfaces, are a significant source of morbidity, including contributions to pelvic pain and female infertility due to distortions in pelvic organ anatomy (1). Postoperative adhesion formation occurs in an average of 85% of patients undergoing abdominal surgery, with an incidence of 55%–100% reported in the literature (2). Although the process of adhesion formation is incompletely understood, hypoxia, resulting from tissue injury, seems to be a key mechanistic factor, triggering a cascade of responses that leads to the development of postoperative adhesions (3). Specifically, hypoxia-mediated production of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$), seems to be critical in the development of postoperative adhesions (4).

Fibroblasts isolated from postoperative adhesions are intimately involved in the process of adhesion development. Such fibroblast have been shown to exhibit a distinct molecular phenotype when compared with

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fibroblasts isolated from normal peritoneal tissue (5), and this finding has been corroborated in numerous animal and human studies (6–9). Utilizing a fibroblast culture system, the effect of hypoxia on the development of the adhesion phenotype has been modeled in vitro, whereby normal human peritoneal fibroblasts exposed to hypoxia have been shown to manifest phenotypic changes characteristic of fibroblasts isolated from peritoneal adhesions (10). Furthermore, acquisition of the adhesion phenotype by these fibroblasts seems to be irreversible (10). The effects of hypoxia on adhesion development are mediated, in part, by hypoxia-inducible transcription factors (HIFs), specifically HIF-1α, which are up-regulated under hypoxic conditions (3, 11).

2,4-Dinitrophenol (DNP) is a lipophilic weak acid that decreases mitochondrial adenosine triphosphate (ATP) production by uncoupling oxidative phosphorylation (12). 2, 4-Dinitrophenol, therefore, decreases ATP production by a mechanism distinct from hypoxia-induced anaerobic glycolysis. We hypothesized that uncoupling oxidative phosphorylation with DNP would promote development of the adhesion phenotype in fibroblasts isolated from normal peritoneal fibroblasts, even in the absence of hypoxia. To test our hypothesis, we measured the expression of type I collagen, vascular endothelial growth factor (VEGF), and HIF-1α, known molecular markers of the adhesion phenotype, in normal peritoneal and adhesion fibroblasts with and without exposure to DNP (5).

MATERIALS AND METHODS

Source and Culture of Human Fibroblasts

Cultures of fibroblasts acquired from normal parietal peritoneal and adhesion tissue of patients undergoing laparotomy for pelvic pain were performed and characterized as previously described (5). Each patient served as her own control. Briefly, normal parietal peritoneal tissue was collected from the anterior abdominal wall lateral to the midline incision at a minimum of 7.6 cm from any visible adhesions. The patients did not have active pelvic or abdominal infections and were not pregnant at the time of surgery. All patients gave informed written consent for tissue collection, which was conducted under a protocol approved by the institutional review board of Wayne State University.

Harvested tissue samples were immediately placed in standard media (Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 2% penicillin, and streptomycin). Tissues were cut into small pieces in a sterile culture dish and transferred into a fresh T-25 flask with 3 mL of dispase solution (2.4 U/mL; GIBCO BRL, Life Technologies). The flasks were incubated overnight at 37°C in an environ-shaker (LAB LINE Instruments). The samples were then centrifuged for 5 minutes at 1,400 $\times$ *g*, transferred into a fresh T-25 flask with prewarmed Dulbecco's Modified Eagle Medium, and placed in a 37°C incubator (95% air, 5% CO_2). Outgrowth of fibroblasts generally took 2 weeks. Once confluence was reached, the cells were transferred to 90-mm^2 tissue culture dishes and were cultured in standard media with 10% fetal bovine serum. Thereafter, the confluent dishes were subcultured by trypsinization (1:3 split ratios). Studies were conducted using passage of three to five cells to maintain comparability.

Treatment of Normal Peritoneal and Adhesion Fibroblasts with DNP

Normal peritoneal and adhesion fibroblasts (5.0×10^6) were treated with or without 0.2 mM DNP (Sigma-Aldrich) for 24 hours. This concentration was selected on the basis of preliminary dose–response studies conducted in our laboratory, which demonstrated a maximum increase in type I collagen levels in normal peritoneal fibroblasts at 0.2 mM DNP. All experiments were performed in triplicate from fibroblasts cultured from three patients.

RNA Isolation and Reverse Transcription

Ribonucleic acid was extracted using the RNeasy Mini Kit (Qiagen) according to the protocol provided by the manufacturer. A 3-μg cDNA reaction was then prepared using the QuantiTect reverse transcription kit (Qiagen).

Real-Time Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Quantitative real-time RT-PCR was performed with the QuantiTect SYBR Green RT-PCR kit (Qiagen) and the SmartCycler System (Cepheid). The PCR mix (25 μL) consisted of 12.5 μL of 2$\times$ QuantiTect SYBR Green RT-PCR master mix, 1 μL of complementary DNA template, and 0.2 μmol/L of target-specific primer that was designed to amplify part of the gene of interest. Optimal oligonucleotide primer pairs were selected with the aid of the Beacon Designer software program (Premier Biosoft International) (Table 1). A standard with a known concentration was designed specifically for type I collagen, VEGF, and HIF-1α using the Beacon Designer software. This allowed for absolute quantification of type I collagen, VEGF, and HIF-1α expressed as copy numbers per femtogram of RNA. After real-time RT-PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product as a single peak. A control, which contained all the reaction components except for the template, was included in all experiments.

The PCR conditions were programmed for the gene of interest as follows: an initial cycle performed at 95°C for 900, 1,000, or 1,200 seconds for type I collagen, VEGF, and HIF-1a, respectively, followed by 35 cycles of denaturation at 95°C for 15 seconds, and annealing for 30 seconds at 58°C, 57°C, or 60°C for type I collagen, VEGF, and HIF-1a, respectively. The final cycle was 72°C for 30 seconds to allow completion of product synthesis. Gene expression was analyzed using SmartCycler Software version 1.2f (Cepheid).

Statistical Analysis

Paired *t* tests were used to analyze differences between treatment groups. Statistical analysis was performed using SPSS version 19.0 for Windows. Significance was defined as $P < .05$.